Journal of Saudi Chemical Society (2015) **xxx**, xxx–xxx

King Saud University

Journal of Saudi Chemical Society

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Preparation and characterization of zinc glycerolate: UV protection, biological activity and permeation study

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Received 4 September 2014; revised 1 September 2015; accepted 13 September 2015

KEYWORDS

Zinc complex;
Zinc glycerolate;
Antimicrobial;
Permeation study

Abstract Zinc glycerolate, has been reported to be beneficial in the treatment of many medical ailments but its properties for skincare application are not explored. Zinc glycerolate has been successfully synthesized by heating zinc oxide and glycerol in 240 °C via microwave irradiation. The characterization and properties of zinc glycerolate were discussed including scanning electron microscopy (SEM), laser diffraction particle size analysis, *in vitro* irritancy potential, UV protection, antibacterial and permeation properties via Franz diffusion cell of the zinc glycerolate. Zinc glycerolate is classified as non-irritant when used in dermal application. It has SPF of 1.007 ± 0.004 and ultraviolet A to B (UVA/UVB) ratio of 0.7 ± 0.019 which is considered good for UVA protection under Boot's star rating. The antimicrobial properties of zinc glycerolate were expressed as the minimum inhibitory concentration (MIC); the minimum bactericidal concentration (MBC) and the time needed to eliminate 99.9% of the bacteria population (time-kill). Zinc glycerolate has better bactericidal properties than zinc oxide particularly towards *Staphylococcus epidermis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Propionibacterium acnes* while no effect was observed for *Candida albicans* and *Aspergillus niger*. The concentration of zinc ions that has diffused through polysulfone membrane installed in a vertical diffusion cell was determined through atomic absorption spectroscopy (AAS). The highest concentration of zinc ion diffused was found from incorporation of zinc glycerolate in oil-in-water (O/W) cream-based excipient. The obtained results indicated that zinc glycerolate has a good potential for applications in the cosmetics and pharmaceutical products.

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Peer review under responsibility of King Saud University.

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1. Introduction

Crystalline metal complexes can be formed by heating certain metal oxides, hydroxides or salt with glycerol at temperature above 110 °C. The metals include cobalt, iron, aluminium

http://dx.doi.org/10.1016/j.jscs.2015.09.002

substituted iron, manganese and zinc [1–7]. Likewise, zinc glycerolate is formed by heating zinc oxide, zinc acetate or zinc hyrozincite with glycerol from 110 to 300 °C by conventional heating or microwave irradiation [8–15]. The empirical formula for zinc glycerolate complex is $(C_3H_6O_3Zn)_x$ [8]. It is proposed in a myriad of applications from cosmetics [8], nutrition as dietary supplement [9,16] to bonding agent in plastic or rubber materials [10]; but most notably in pharmaceutical [8,9,18–21].

Zinc is known as essential dietary micronutrient with beneficial functions that facilitate cytoprotection and tissue repair in humans [22]. Subcutaneous, percutaneous and intramuscular deposition of zinc glycerolate are useful in the treatment of local skin disorders [8,16], zinc deficiency [16], systemic inflammatory diseases like arthritis [16,17], oral herpetic sores [18], while oral administration of this complex aids the healing of gastric ulcers (gastroprotective agent) [9,16] and diabetes [19–21].

The potential applications of zinc glycerolate in cosmetics were suggested but very much less explored. Application of zinc glycerolate can be quite similar to zinc oxide. It can be physically retained on the skin healing with minimal or no skin irritation to provide antioxidant photoprotection to the skin [23], protect the skin against UV-induced skin damage, such as sunburn, cancer, premature ageing, photoallergies [8] and prevent microbial proliferation [9]. Its antimicrobial activities are similar with other zinc complexes such as zinc chloride, zinc oxide, zinc acetate, zinc phosphate and zinc polycarboxylate [24–41]. Therefore, we envisaged that zinc glycerolate is able to exert its antimicrobial properties by liberation of Zn^{2+} by hydrolysis.

In our previous work, we have synthesized and characterized the physical properties of zinc glycerolate. We found that zinc glycerolate hydrolyses easily in aqueous condition and can be minimized by incorporation of zinc glycerolate with 6.5 M glycerol solution [42].

The aim of this work is mainly to evaluate the biological properties of zinc glycerolate and assess the feasibility of using the complex in the cosmetics and personal care. The dermal irritancy potential of zinc glycerolate was established by *in vitro* methodology while the sun protection factor (SPF) was determined *via* an ultraviolet transmittance analyser. The antimicrobial activity of the zinc complex was determined by a susceptibility test, MIC, MBC and a time kill study. Zinc glycerolate in cream was used in Franz-type diffusion cells with polysulfone membrane as a model to assess the bioavailability of zinc ions into the human epidermis.

2. Experimental

2.1. Chemicals and reagents

Zinc oxide (ZnO) is the most notable of zinc derivatives and is widely used in cosmetics as a sunscreen agent hence will be used as the benchmark in this study. Zinc oxide supplied as zinkoxid reinst (ZnO) was obtained from Merck (Darmstadt, Germany) while vegetable oil based glycerine (Pricerine™ 9091) were obtained from Croda Inc. Acetone was obtained from J.T. Baker (U. S. A.) to be used in the purification of the samples. All other reagents were of analytical grades and used as received.

Crodacol™ CS90EP, Crodamol™ GTCC, Crodamol™ IPM, Arlacel™ 165, Cithrol™ GMS 40 and Pricerine™ 9091 were obtained from Croda International Plc (UK) while Euxyl® PE 9010 were brought from Schülke & Mayr (Norderstedt, Germany). Petrolatum (Vaseline®) was purchased from Sigma–Aldrich.

2.2. Bacterial strains and medium

The modified susceptibility test, minimum bacterial concentration evaluation and time kill study utilized Mueller–Hinton agar which was obtained from Oxoid (U. K.). Sabaroud dextrose agar (SDA), Mueller–Hinton broth and potato dextrose broth were purchased from Difco (U. S. A.). American Type Culture Collection (ATCC) bacterial strains used were *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 15442, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 9642, *Staphylococcus epidermis* ATCC 12228 and *Propionibacterium* acnes (clinical isolates obtained from the Culture Collection Centre, Bacteriology Unit, Institute for Medical Research, Malaysia).

2.3. Preparation and characterization of zinc glycerolate

Zinc oxide and glycerol at a molar ratio of 1:3 were heated with frequent agitation using an Ethos MR Microwave Reactor (Milestone, Germany). The initial duration of heating and the temperature was set at 10 min and 40 °C, respectively. The optimum temperature was set at 250 °C and the retention time at this temperature was varied from 20 min to 1 h. The venting time was set at 10 min. The reaction mixture was left to cool and later purified using acetone. The slurry was filtered using a Buchner funnel lined with filter paper No. 1. The sample was dried in an oven at 90 °C for 20 min. The morphology of the zinc glycerolate particles were scanned by a Philips XL30 scanning electron microscope. The particle size of dispersed zinc glycerolate was obtained by laser diffraction size analysis using a Malvern Mastersizer Hydro 2000S particle size analyser. The result of particle size was taken in triplicate.

2.4. In vitro dermal irritection

All potential cosmetics ingredient must be tested to ensure no skin irritation when used. The principle of the testing is as follows: dermal irritancy induced by any substance or ingredient is related to its ability to denature and disrupt corneal proteins or alter the structure of keratin, collagen and other dermal proteins. The dermal irritection assay [43], an alternative method to animal irritancy tests or Draize test, correlates well with *in vivo* irritancy tests [44]. The dermal irritection assay kit from *in vitro* International is used as supplied. The dermal irritection assay requires two components: a membrane substrate modified by covalently cross linking a mixture of keratin, collagen and an indicator dye, and a reagent solution consisting of an organized globulin/protein matrix. Application of an irritant chemical to the membrane disc disrupts the ordered structure of keratin and collagen and results in release of the bound indicator dye. Additionally, dermal irritants induce conformation changes in the globular proteins found in the reagent solution. The extent of dye release and protein denaturation may be quantitated by measuring the changes in optical density of the reagent solution at 450 nm (OD_{450}). Comparison of optical density to those produced by standard chemical irritants and those produced by the test material

Please cite this article in press as: M.Y. Cheong et al., Preparation and characterization of zinc glycerolate: UV protection, biological activity and permeation study, Journal of Saudi Chemical Society (2015), http://dx.doi.org/10.1016/j.jscs.2015.09.002

permits calculation of an irritancy score that has been shown to be directly related to the dermal irritancy of the test material. The dermal irritancy potential is expressed as the human irritancy equivalent (HIE) scores. The predicted *in vivo* classifications based on these scoring systems are shown in Table 1.

Samples were made up at five different concentrations: 25, 50, 75, 100 and 125 mg. These were placed into the membrane discs. Reagent and blanking buffer (1250 μL) were added to a 24-well assay plate. The membrane discs that contained various concentrations of zinc glycerolate samples were inserted into the corresponding blank and test sample wells of the plate. The assay plate was then incubated at 25 °C for 24 h. The membrane discs were then removed from the assay plate, subsequently 250 μL of reagent and blanking buffer were transferred into the 96-well reading plate. The plate was then inserted into the MRX Microplate Reader from Dynex Technologies, Inc. (Chantilly, VA).

2.5. In vitro SPF

A Labsphere Ultraviolet Transmittance Analyzer UV-1000S was used to evaluate the *in vitro* SPF value of zinc glycerolate based on their recommended procedure in line with ISO 24443 [45]. 3M Transpore™ tape was used as the substrate for *in vitro* SPF measurement because of its transparency to the ultraviolet light. 3M Transpore™ tape was placed in a single layer on a clean 2 mm thick quartz slides. An area of at least two square inches (12.5 cm^2) was used to enable measurement over at least five non-overlapping spots. Samples of 5.0%, 10.0% and 20.0% of zinc glycerolate, titanium dioxide or zinc oxide were prepared by mixing with glycerol to form a paste. About 2.0 mg/cm^2 samples were distributed evenly over the entire surface of the sample plate. The samples were put aside to dry for at least 20 min before measurements were made. Twelve readings were taken for each sample. The testing of samples was performed by running a baseline on the reference media of glycerol before the prepared samples were analysed.

SPF by definition is determined *in vivo* as the increase in exposure time required to induce erythema. However, the *in vitro* technique involves measuring the spectral transmittance at UV wavelengths from 280 nm to 400 nm. The *in vitro* SPF is calculated based on Eq. (1):

$$\mathrm{SPF} = \frac{\int_{280\mathrm{nm}}^{400\mathrm{nm}} E_\lambda . S_\lambda d\lambda}{\int_{280\mathrm{nm}}^{400\mathrm{nm}} E_\lambda . S_\lambda . T_\lambda . d\lambda} \quad (1)$$

where, E_λ = CIE erythemal spectral effectiveness, S_λ = solar spectral irradiance, T_λ = spectral transmittance on the sample (as measured on the UV-1000S).

The equation shows that the higher the amount of transmittance, the lower the SPF value. The transmittance spectrum of a sunscreen in either region is averaged in order to produce a single value, which describes the amount of UV-A or UV-B blocking. The average transmittance in each region is given by Eqs. (2) and (3) respectively:

$$\mathrm{T(UVA)_{av}} = \frac{\sum_{315\mathrm{nm}}^{400\mathrm{nm}} T_\lambda \Delta_\lambda}{\sum_{315\mathrm{nm}}^{400\mathrm{nm}} \Delta_\lambda} \quad (2)$$

and

$$\mathrm{T(UVB)_{av}} = \frac{\sum_{280\mathrm{nm}}^{315\mathrm{nm}} T_\lambda \Delta_\lambda}{\sum_{280\mathrm{nm}}^{315\mathrm{nm}} \Delta_\lambda} \quad (3)$$

where, Δ_λ = measured wavelength interval.

Table 1 Classification of human irritancy equivalent (HIE) score to predicted *in vivo* irritancy dermal [43].

Human irritancy equivalent	Predicted dermal irritancy classification
0–0.90	Non-irritant
0.90–1.20	Non-irritant/irritant
1.20–5.00	Irritant

Consequently, the percent blocking for UVA and UVB, respectively, is calculated as in Eq. (4);

$$100\% - \mathrm{T(UVA)_{av}} \text{ or } 100\% - \mathrm{T(UVB)_{av}} \quad (4)$$

where, $\mathrm{T(UVA)_{av}}$ or $\mathrm{T(UVB)_{av}}$ are expressed as a percentage.

In addition to its ability to determine the SPF of a sunscreen, the *in vitro* technique can also measure the UVA protection of the sunscreen. Boots the Chemist, the largest producer of sunscreens in the UK, has developed a label system that uses star ratings for protection based on spectrophotometric analysis. The Boots star rating system is a proprietary *in vitro* method used to describe the ratio of UVA to UVB protection offered by sunscreen creams and sprays as classified in Table 2.

The spectral transmittance values, T_λ, are converted to spectral absorbance values, $A_\lambda = -\log(T_\lambda)$. A term called the UVA ratio is calculated, which is the ratio of the total absorption in the UVA to that in the UVB as in Eq. (5).

$$\frac{\mathrm{aUVA}}{\mathrm{aUVB}} = \frac{\int_{280\mathrm{nm}}^{400\mathrm{nm}} A_\lambda d\lambda}{\int_{290\mathrm{nm}}^{320\mathrm{nm}} A_\lambda . d\lambda} \quad (5)$$

2.6. Susceptibility testing

In order to test the zinc glycerolate which is an insoluble powder in water, a modified Kirby–Bauer method was used for susceptibility testing [46]. The modification consisted of utilizing a sterile, stainless steel cork-borer with circular diameter of 5 mm to bore sample wells on Mueller–Hinton agar that had been swiped three times with a 0.5 McFarland standard suspension of microorganisms with sterile cotton buds. The wells were sealed with one drop of sterile molten agar to prevent leakage from the base of the seeded culture plates. The following monoculture strains have been used for streaking the surface of Mueller–Hinton agar: *S. aureus*, *E. coli*, *P. aeruginosa*, *C. albicans*, *A. niger*, *S. epidermis* and *P. acnes*. All the aerobic bacteria were grown to confluence on Mueller–Hinton agar (Oxoid) for 24 h at 36 °C and harvested into

Table 2 The Boots star rating and classification for UVA protection is determined from the measured UVA to UVB ratios [45].

UVA Ratio	Star category	Category descriptor
0.0 to < 0.2	*	Too low for UVA claim
0.2 to < 0.4	**	Moderate
0.4 to < 0.6	***	Good
0.6 to < 0.8	****	Superior
⩾0.8	*****	Ultra

The star category denotes the protection level of the sunscreen. It was first developed by Boots and known as Boots Star Rating System.

0.9% sterile saline solution except *C. albicans* and *A. niger* which were grown to confluence on SDA for 72 h at 25 °C. *P. acnes* was grown at Columbia sheep blood agar in an anaerobic jar with anaerobic atmosphere generation bags for 24 h at 36 °C. Samples were harvested into 0.9% sterile saline solution and then standardized to 10^8 colony forming units (CFU) per millilitre by turbidity or equivalent to a 0.5 McFarland turbidity standard. It was verified by a UV–VIS spectrophotometer at an optical density of 600 nm absorbance of approximately 0.08 0.10. For *A. niger*, a haemocytometer was used to adjust the density of spores to 1×10^8 CFU/mL.

Approximately 0.01 g of sample was placed in the cut-out sample wells. The agar plates were incubated at 36 °C for 24 h for aerobic bacteria and anaerobically for *P. acnes*. The incubation condition for *C. albicans* and *A. niger* was 25 °C for 72 h. The diameter of the zone of inhibition of the samples tested was compared with a 10 μg streptomycin disc for bacteria and 10 μg miconazole for *C. albicans* and *A. niger*.

2.7. MIC and MBC determination

A macrodilution susceptibility testing method was used to determine the minimal concentration of antimicrobial agent needed to inhibit or kill *S. aureus*, *E. coli*, *P. aeruginosa*, *C. albicans*, *A. niger* and *S. epidermis* [47]. Zinc glycerolate, from 0.01 g to 0.20 g in 0.01 g increments, was placed in sterile test tubes and then 1.8 mL of sterile Mueller–Hinton broth was placed into the test tubes with 0.2 mL of standardized 0.5 McFarland suspensions of bacteria. The same procedure was repeated for *C. albicans* and *A. niger* except Mueller–Hinton broth was replaced by potato dextrose broth. For *A. niger*, a haemocytometer was used to adjust the density of spores to 1×10^8 CFU/mL. The broth was inoculated to a final cell density of 1×10^6 CFU/mL. The test tubes were incubated at 36 °C for 24 h for bacteria or at 25 °C for 72 h for *C. albicans* and *A. niger*. After incubation, the lowest concentration of zinc glycerolate showing no visible growth was considered as the MIC [47]. MBC was determined by pipetting 0.1 mL of the inoculated Mueller–Hinton or potato dextrose broth onto Mueller–Hinton agar for bacteria or SDA for yeast and fungi. The plates were then incubated at 36 °C for 24 h for bacteria or 25 °C for 72 h for *C. albicans* and *A. niger*. Colony counts were made after incubation and the surviving colonies were compared to the starting inoculum. The lowest concentration of sample required to kill 99.9% the tested microorganism was recorded as MBC. If the material being tested is bacteriostatic, there will be bacterial colonies observed upon incubation after plating while no positive growth will be seen if the material is bactericidal. The same principal applied to yeast and fungi to establish if the material is fungicide or fungistatic [41].

2.8. Time-kill study

The time kills study was done in a manner as described [48]. The selected bacteria, yeast and fungi strains used in the study were *S. aureus*, *E. coli*, *P. aeruginosa*, *C. albicans*, *A. niger* and *S. epidermis*. A 0.1 mL suspension of bacteria was standardized to 0.5 McFarland and inoculated in 0.9 mL Mueller–Hinton broth supplemented with 0.05 g zinc glycerolate so that the broth was inoculated to a final cell density of 1×10^6 CFU/mL Aliquots of 0.1 mL were taken at hourly intervals, serially diluted, plated on Muller–Hinton agar and incubated at 37 °C for 24 h to determine total viable counts. The same procedure was repeated for yeast and fungi strains by replacing Mueller–Hinton broth with potato dextrose broth and SDA and incubating at 25 °C for 72 h. Kill curves were constructed by plotting $\log_{10}$ CFU against time. Testing for neutralization of the sample was done to ensure no false negative was recorded due to presence of active zinc complex carried over from the sample into the media which may inhibit viable microorganisms. Determination of the initial number of viable microorganisms was done by running a control test using sterile deionized water instead of the zinc glycerolate.

2.9. Preparation of O/W emulsion

Non-ionic O/W emulsions were prepared: zinc glycerolate cream with 5% w/w of zinc glycerolate, zinc oxide cream with 10% w/w of zinc oxide and placebo cream. To prepare the cream, the oily (Crodacol™ CS90EP, Crodamol™ GTCC, Crodamol™ IPM, Arlacel™ 165 and Cithrol™ GMS 40) and aqueous phases (Pricerine™ 9091 and deionized water) were heated separately until reach 75 °C, then the oily phase was added to the aqueous phase and the system was mixed (Polytron, 500 rpm) with constant agitation for 5 min before stirring was slowed to 150 rpm. Preservative (Euxyl® PE 9010) and zinc complex (zinc glycerolate or zinc oxide) were added into the cream when the temperature reached 25 °C.

2.10. Permeation study by Franz diffusion cell

The permeation of chemicals through the skin can be measured by *in vitro* techniques using the static, non-flowing cell generally known as Franz diffusion cell. Permeation test through polysulfone membrane (HT-450 Tuffryn®, Pall) with diameter of 25 mm and pore size of 0.45 μm were carried out using a vertical diffusion cell system (Hanson Research, Chatsworth CA, USA) consisting of six thermostated cells with the lower donor and the upper receptor chambers separated by membrane, the stratum corneum facing the donor chamber. The donor compartment diameter was 15 mm, providing an application area of 1.77 cm^2. This cell has a static receptor solution reservoir with a side-arm sampling port. Permeation was monitored by sampling the stirred receptor chamber solution [49]. Isotonic phosphate buffered saline pH 7.4 (PBS) was prepared by dissolving 8.00 g NaCl, 0.20 g KCl, 0.20 g KH_2PO_4 and 1.44 g $Na_2HPO_4{\cdot}2H_2O$ in 1 L distilled water. PBS was used as the receptor solution. The receptor compartment was filled with 4.3 mL of PBS which was kept at 37 °C by circulating water through an external water jacket. After 30 min of equilibration of the membrane with the receptor solution, 3.0 g of the sample was applied in the donor compartment. The donor compartment was covered with glass slides to prevent evaporation of the solvent. The receptor solution was continuously stirred by means of a spinning bar magnet, at 400 rpm. Receptor solution samples were withdrawn as 2.0 mL aliquots, through the sampling port of the receptor compartment at various time intervals. The experiments were run for 6 h and were repeated three times. The concentration of zinc ions in the receptor solution was determined by means of atomic absorption spectroscopy (AAS) from Thermo Finnigan Model S4.

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2.11. Data analysis

Linear regression analysis of pseudo-steady-state diffusion plots allowed calculation of the following parameters: steady-state flux (J), given by $Q/A.t$, where Q is the amount of permeate diffusing across the area (A) in time (t). Lag time (t_L), indicated the time taken by the sample to saturate the membrane and to reach the receiving phase, calculated from the X-axis intercept values of the regression lines with amount of zinc ion permeated at end of experiment ($Q_{60\ min}$) is calculated. TRPE apparent diffusion coefficient through membrane was calculated according to the following relation: $D_m = h^2/6\text{-}t_L$ where h represents the thickness of the membrane of the membrane and t_L the lag time. The thickness of the membrane was 145 μm, as indicated from the product specification. The permeability coefficient, K_p was calculated using equation: $K_p = J/C_o$ where J is the flux at steady state (mg/cm^2 min) and C_o is the initial zinc oxide or glycerolate concentration (mg/L). Membrane/vehicle partition coefficient, K_m, was obtained from the relationship: $K_p = K_m D_m/h$ [50].

3. Result

3.1. Evaluation of zinc glycerolate

The SEM of zinc glycerolate and zinc oxide is given in Fig. 1a and b, respectively. The zinc glycerolate obtained was a random heap of hexagonal prisms with an average diameter and thickness of ca. 2.5 μm and ca. 350 nm, respectively. The average particle size by volume weighted mean, D (4,3) of zinc glycerolate was 23.015 μm as shown in Fig. 2 while for zinc oxide it was 12.543 μm.

3.2. Evaluation of in vitro irritection and SPF

The dose response curve of HIE scores on dermal for zinc glycerolate is shown in Fig. 3. The graph indicated that even at increasing dosage of zinc glycerolate from 50, 75, 100 to 125 μL, the results were below the threshold value of HIE score of 0.9, hence it is classified as a non-irritant.

Attenuation spectra for 20% zinc glycerolate are shown in Fig. 4 with UVA (320–400 nm) and UVB (290–320 nm). Fig. 5 shows the SPF values, UVA to UVB ratios and the Boots star rating of zinc oxide, titanium dioxide and zinc glycerolate respectively. Generally, average SPF values and UVA to UVB ratios of all samples in glycerol increased in line with the higher sample amount of zinc oxide, titanium dioxide and zinc glycerolate respectively in the formulation. Zinc glycerolate has the lowest SPF value, UVA/UVB ratio and Boots star rating as compared to zinc oxide and titanium dioxide, even at increased concentrations. However it is noted that the Boots star rating of 20% zinc glycerolate is comparable to 5% titanium dioxide. At 5% concentration, zinc glycerolate has a mean SPF of 1.07 ± 0.004 and a UVA/UVB ratio of 0.7 ± 0.019, which is considered 'good' (three stars) under the Boots star rating for UVA protection. Comparatively, the SPF value of 5% titanium dioxide is 1.33 ± 0.005, the UVA/UVB ratio is 0.85 ± 0.036 and has a 'superior' Boots star rating (four stars). The SPF value of 5% zinc oxide is 1.36 ± 0.05, the UVA/UVB ratio is 0.91 ± 0.023 and has an 'ultra' Boots star rating (five stars).

3.3. Evaluation of antimicrobial activity

Fig. 6 shows the zone of inhibition around the 6 mm diameter wells made by the cork-borer filled with 10 μg zinc glycerolate or zinc oxide and 10 μg of streptomycin disc of 6 mm diameter. The diameter of the zones of inhibition recorded in Fig. 7 for bacteria and Fig. 8 for yeast and mould. There were positive zones of inhibition for *S. aureus*, *E. coli*, *S. epidermis*, *P. acnes*, *C. albicans* and *A. niger* while no inhibitory activity were recorded for *P. aeruginosa*.

The results of susceptibility tests with zinc glycerolate against reference strains are summarized in Table 3. The MIC_{50s} and MIC_{90s} were in the range of 5 mg/mL for *S. epidermis* and *S. aureus* while for *E. coli* and *P. aeruginosa* it is 10 mg/mL. The MIC_{50s} in the range of 10 mg/mL for both *C. albicans* and *A. niger* while MIC_{90s} value ranges from 10 to 30 mg/mL for *C. albicans* and *A. niger* respectively. The MBCs were in the range of 5.0–15 mg/mL for bacteria. It was noted that optimum inhibition power of 99.9% population wipeout or MBC was exhibited at 5.0 mg/mL for *S. epidermis* and *S. aureus* while 15.0 mg/mL for *E. coli* and *P. aeruginosa*. Positive growth was observed for *C. albicans* and *A. niger* when the broth was pipetted out and set into agar.

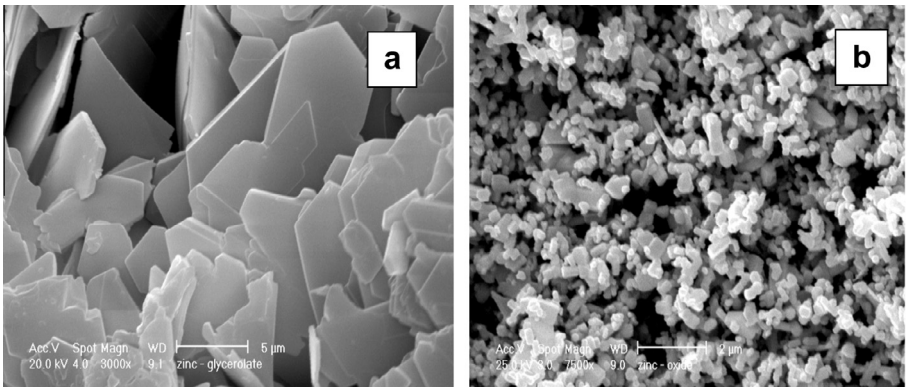


Figure 1 Scanning electron microscopy of (a) zinc glycerolate magnified to 3000× and (b) zinc oxide magnified to 7500×.

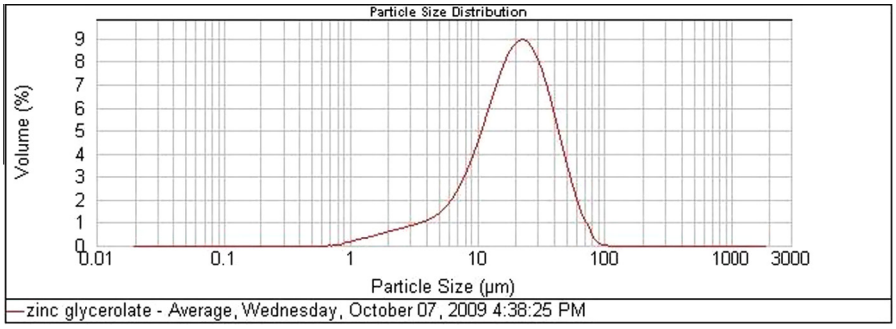


Figure 2 Size distribution of zinc glycerolate particles.

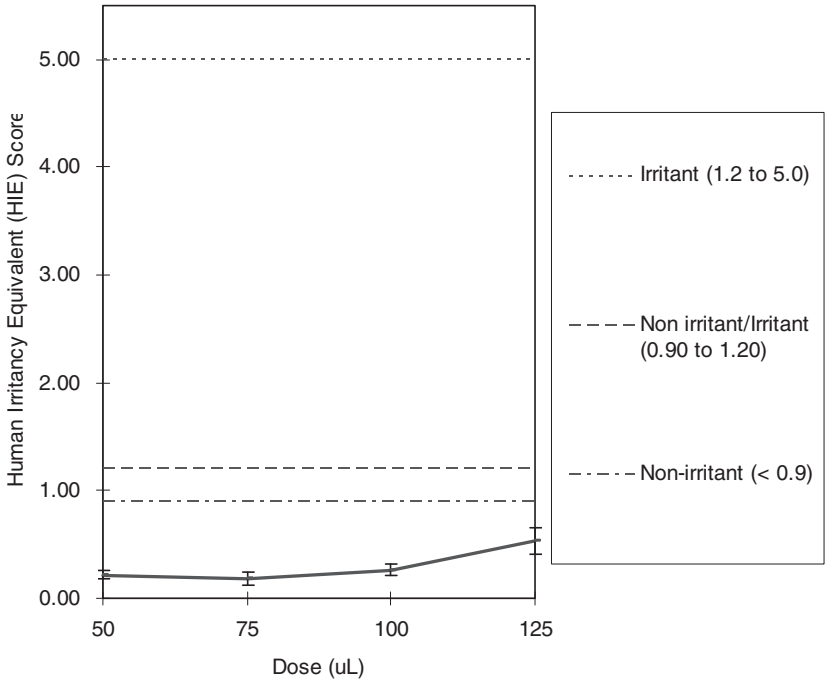


Figure 3 Dose–response curve for HIE score on dermal for zinc glycerolate. The data represent ±SD of three replicates.

As further extension of the MIC and MBC, time kill studies were initiated. Fig. 9(a–d) demonstrate time-dependent microbicidal action of zinc glycerolate at 50.0 mg/mL, which is slightly higher than the minimum MBC needed to inactivate *P. aeruginosa*. The slope of the line of regression (*K*) could be used for the analysis of bactericidal activity of zinc glycerolate against different microbial species [48]. From the analysis of kill curve the time required to achieve 1 $\log_{10}$ reduction (90%) or 3 $\log_{10}$ reduction (99.9%) decrease in viable cell count was calculated (Table 4). Data in Table IV show that the time required to achieve a 1 $\log_{10}$ reduction (90%) in all tested strains (except for yeast and fungi) ranged within 1.19–2.81 h. In general, Gram-positive bacteria were at the lower end and Gram-negative bacteria were at the higher end of reduction range indicating Gram-positive bacteria were killed faster than Gram-negative bacteria. Also, at lower absolute values of *K*, the bactericidal activity was found to be greater [48]. Among all the strains tested Gram-positive bacteria such as *S. aureus* and *S. epidermis* were found to be the most susceptible to zinc glycerolate as 90% of the population were inhibited in the least time in about 1.19 h while Gram-negative bacteria such as *E. coli* and *P. aeruginosa* were inhibited in 1.58 and 2.81 h respectively. The time required to achieve 3 $\log_{10}$ decreases in viability was also determined from the time kill curves at 50.0 mg/mL zinc glycerolate. Once again, zinc glycerolate kills Gram-positive bacteria more effectively, achieving a 3 $\log_{10}$ decrease in 3.57 and 3.68 h for *S. epidermis* and *S. aureus* respectively versus 4.74 and 8.42 h killing time for *E. coli* and *P. aeruginosa* respectively. There was no growth seen after incubation of 8 and 24 h for the tested microorganism when plating out was done except for *C. albicans* and *A. niger* which showed positive growth. Neutralization studies done on all diluted plates indicated no false negatives.

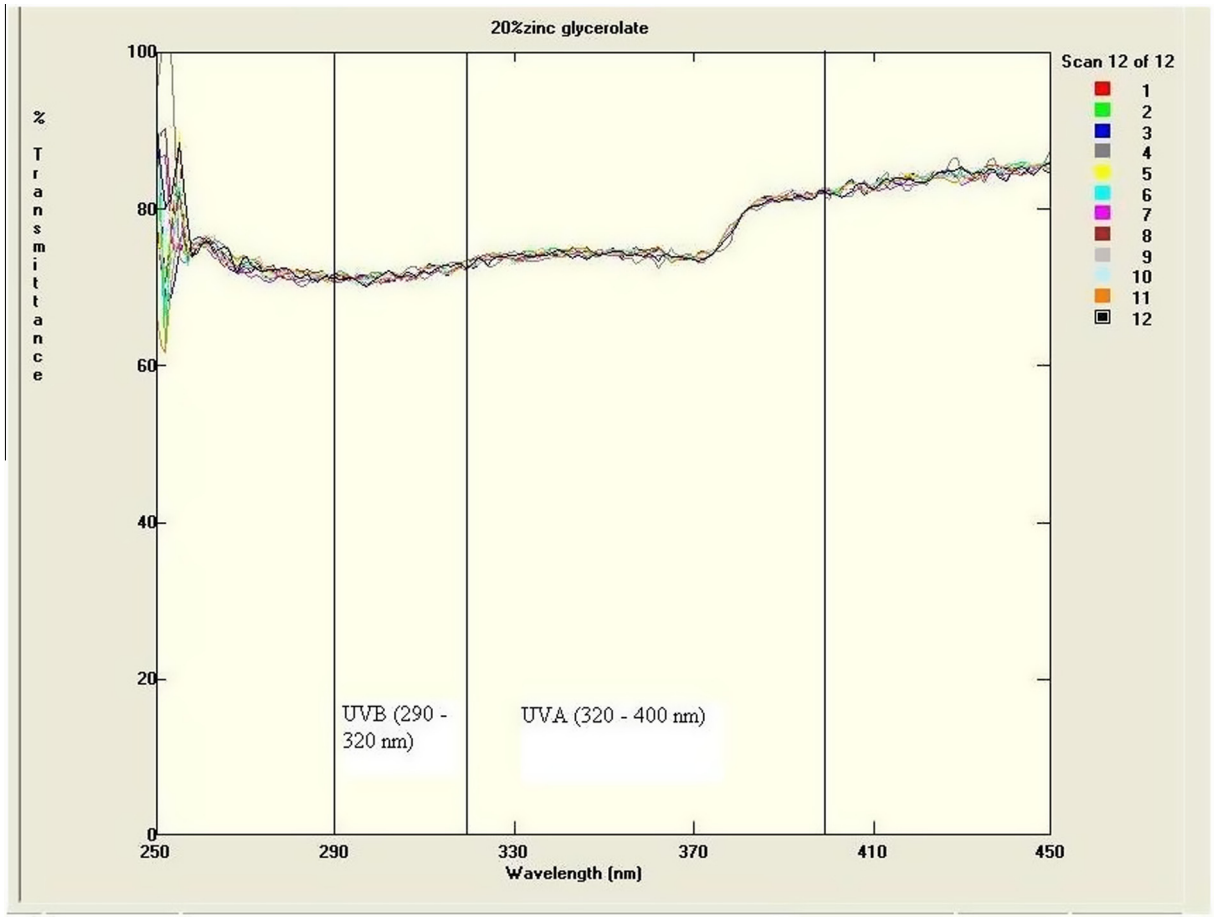


Figure 4 Attenuation spectra for 20% zinc glycerolate in glycerol. Twelve scans were made for each sample.

3.4. Evaluation of zinc permeation

The ability of zinc ions to permeate the human skin as modelled using a polysulfone membrane with PBS as the receptor solution. The samples used were 50.0 mg/mL zinc glycerolate in petrolatum base, 50.0 mg/mL zinc oxide powder in petrolatum base and 50.0 mg/mL zinc glycerolate powder in cream base which consisted of a propriety combination of medium chain triglycerides, wax, glycerol and emulsifiers. As indicated in Fig. 10, the highest zinc level was detected from zinc glycerolate in a cream base throughout the experiment compared to pure zinc glycerolate and zinc oxide powder.

Results of zinc ion permeation experiments are illustrated graphically in Fig. 10, whereas the relevant permeation parameters (J, t_L, $Q_{60\ \mathrm{min}}$, K_p, D_m, K_m) are summarized in Table 4. The permeation profiles demonstrated that the incorporation of the O/W emulsion with 5% (w/w) zinc glycerolate remarkably increased the permeation of zinc ion through membrane. After application of the mixture above, the steady state flux of zinc ion through membrane was found to be 0.0017 mg cm^{-2} min^{-1}. The permeation of zinc from petrolatum as excipient decreased about three times with 0.00051 μg cm^{-2} min^{-1} flux. In addition, the amount of zinc ion permeated from O/W emulsion was 1.10 ± 0.08 mg cm^{-2} compared to 0.33 ± 0.02 mg cm^{-2} obtained from 5% (w/w) zinc glycerolate in petrolatum mixture. In all cases, the differences were statistically significant between 5% (w/w) zinc glycerolate in two different kind of excipient; O/W emulsion and petrolatum: $P = 0.013$ in the case of flux; $P = 0.036$ for zinc ion permeated. It is confirmed that the rate-limiting step was the zinc ion release from the formulation as the water in the O/W influences the zinc ion availability for hydrolysis. Petrolatum's hydrophobicity which coated zinc glycerolate and rendered insoluble with the water minimized the hydrolysis of zinc glycerolate [50].

4. Discussion

The SEM showed the differences between zinc glycerolate and zinc oxide. The large, plate-like and hexagonal shape of the zinc glycerolate complex was well defined; which distinguished it easily from the more regular and fine particulates of zinc oxide. Zinc glycerolate which was formed had a substantially greater development in two dimensions in one plane than in a third dimension normal to the other plane. The lustrous, large sized crystallite particles endowed zinc glycerolate with a high lubricity which is an advantageous attribute for topical dermal application [9].

Although zinc glycerolate is classified as non-irritant to dermal application and regarded as safe for use in cosmetics products, an *in vivo* study needs to be conducted to establish its irritancy potential in human living systems. The lower values of SPF, UVA/UVB ratio and Boots star rating of zinc

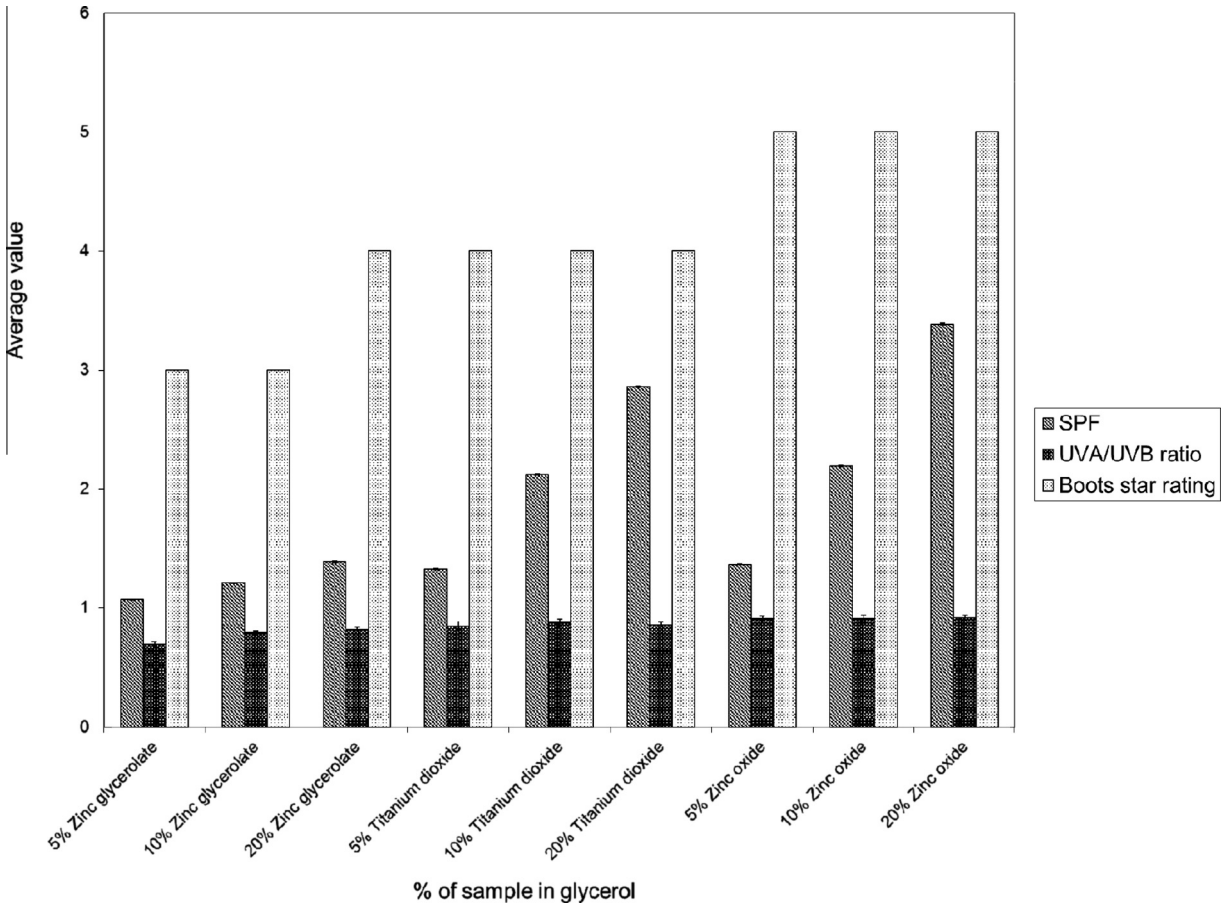


Figure 5 Average SPF values, UVA to UVB ratios and Boots star rating of zinc glycerolate, titanium dioxide and zinc oxide. The data represent ±SD of twelve scans.

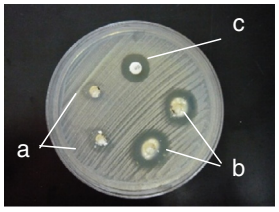


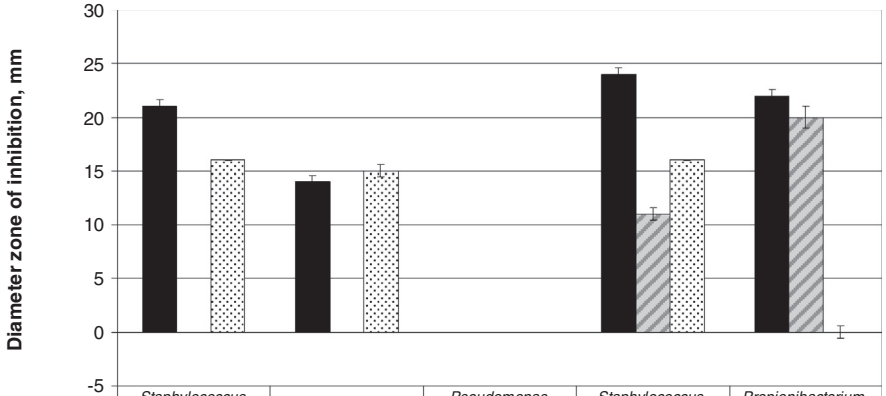
Figure 6 Antimicrobial susceptibility testing of zinc glycerolate and zinc oxide with streptomycin as a control on an agar plate swiped with *S. aureus*, well (a) contains 10 μg of zinc oxide, well (b) has 10 μg of zinc glycerolate and well (c) has 10 μg of streptomycin.

glycerolate as compared to zinc oxide could be attributed to the huge size of zinc glycerolate as compared to finer particulate of zinc oxide as per result from laser diffraction size analysis. This could be because of the presence of trace glycerine which rendered the powder sticky and hence the particulates agglomerated. It is assumed that absorption, scattering and reflection become insignificant when the size of the mineral particles becomes coarse. Such mineral sunscreens also have poor UV protection performance because light 'leaks' through the gaps between large particles.

Positive zones of inhibition were observed for *S. aureus*, *E. coli*, *S. epidermis*, *P. acnes*, *C. albicans* and *A. niger* in the susceptibility testing using a modified Kirby–Bauer method. Though the result is positive, the size of the zone must not be interpreted as a quantitative evaluation of antimicrobial activity [47]. Zinc glycerolate exhibits better bacteriostatic activity against selective bacteria and better fungistatic activity towards yeast and mould than zinc oxide. It was also noted that Gram-positive bacteria were susceptible while Gram-negative bacteria were quite resistant to zinc complexes tested.

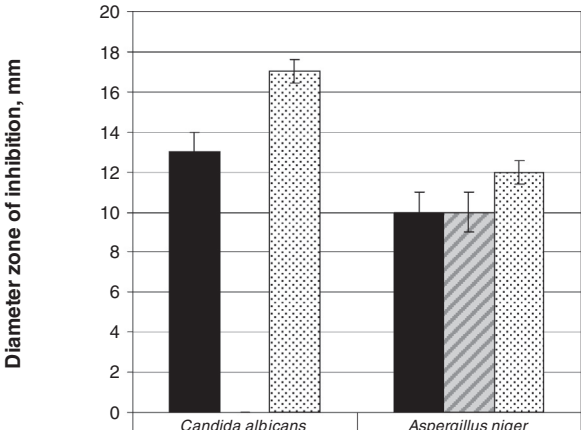
The MIC via the broth dilution method showed that zinc glycerolate has bacteriostatic activity towards *S. epidermis*, *S. aureus*, *E. coli* and *P. aeruginosa* while fungistatic effect was observed for *C. albicans* and *A. niger*. However the MBC result showed that zinc glycerolate has bactericidal activity towards *S. epidermis*, *S. aureus*, *E. coli* and *P. aeruginosa*. It showed fungistatic activity that prevents proliferation of *C. albicans* and *A. niger* as indicated by the MBC result. There were no fungicidal or sporicidal effects when positive growth of these yeast and fungi were observed when the broth was plated out [41].

In time-kill study, the decrease in viability in 8 h and no positive growth upon continued incubation for 24 h had confirmed that zinc glycerolate possess bactericidal effect. The time-kill study also reconfirmed that zinc glycerolate has fungistatic activity against *C. albicans* and *A. niger* when positive growths were observed for both yeast and fungi respectively after the tested broth was plated out. The antimicrobial effect



	Staphylococcus aureus	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus epidermis*	*Propionibacterium acnes*
Zinc glycerolate, 10 μg	21	14	0	24	22
Zinc oxide, 10 μg	0	0	0	11	20
Streptomycin, 10 μg	16	15	0	16	0

Figure 7 Antimicrobial susceptibility testing of zinc glycerolate and zinc oxide with streptomycin as control. When there are positive zones of inhibition, the diameter including the 6 mm diameter of the wells or discs were recorded while none if no zone of inhibition seen. The data represent ±SD of three replicates.



	Candida albicans	*Aspergillus niger*
Zinc glycerolate, 10 μg	13	10
Zinc oxide, 10 μg	0	10
Miconazole, 10 μg	17	12

Figure 8 Antimicrobial susceptibility testing of zinc glycerolate and zinc oxide with miconazole as control. When there are positive zones of inhibition, the diameter including the 6 mm diameter of the wells or discs were recorded while none if no zone of inhibition seen. The data represent ±SD of three replicates.

of zinc glycerolate against the tested microorganisms present in the following order:

Staphylococcus epidermis ATCC 12228 < *Staphylococcus aureus* ATCC 6538 < *Escherichia coli* ATCC 8739 < *Pseudomonas aeruginosa* ATCC 15442

Divalent cations are essential nutrients for bacteria and are required as trace elements at nanomolar concentrations. *In vitro* bacterial growth requires zinc ion concentrations of 10^{-5}–10^{-7} M for optimal growth [35]. However, at higher concentrations, these ions are potentially toxic. The toxicity of heavy metals is mainly due to their interference with microbial metabolism or their altering of the physicochemical environment of cells [51]. A concentration of 10 mM Zn^{2+} decreased the survival of *E. coli* but did not affect that of *P. aeruginosa* after 3 h of incubation in buffer [52].

The antibacterial activity of the Zn^{2+} ion could be due to transmetallation, in which zinc glycerolate complex exchanges zinc for other vital metals in bacterial cells [53]. Zinc glycerolate has weak a metal-bidentate ligand bond via its 1,2-hydroxyl groups that results in fast hydrolysis of this complex in aqueous conditions resulting in zinc ions and glycerol that diffuse into the agar. The liberation of low-molecular weight components like glycerol, zinc ions and $Zn(OH)^{+}$ species may have provided the inhabitable environment for tested bacteria, yeast and fungi to proliferate. On the other hand, zinc ions could be toxic to bacteria though the mechanism for the toxicity of Zn^{2+} is undefined although several possible mechanisms have been proposed. Excess of Zn^{2+} may interfere with the metabolism of Mg^{2+} [54,55]. Bacterial cells take in zinc

Table 3 The MIC and MBC of zinc glycerolate against different organisms.

Organism used	Zinc glycerolate (mg/mL)		
	MIC^{a}_{50}	MIC^{b}_{90}	$MBC^{c}_{99.9}$
Staphylococcus epidermis ATCC 12228	5.0	5.0	5.0
Staphylococcus aureus ATCC 6538	5.0	5.0	5.0
Escherichia coli ATCC 8739	10.0	10.0	15.0
Pseudomonas aeruginosa ATCC 15442	10.0	10.0	15.0
Candida albicans ATCC 10231	10.0	10.0	ND
Aspergillus niger ATCC 9642	10.0	30.0	ND

MIC^{a}_{50}: minimum inhibitory concentration required to inhibit the growth 50% of population (scored visually after 14–16 h of incubation). MIC^{b}_{90}: minimum inhibitory concentration required to inhibit the growth 90% of population (scored visually after 24 h of incubation). $MBC^{c}_{99.9}$: minimum bactericidal concentration required to kill 99.9% of population (scored by spot inoculation on Muller–Hinton agar from the wells showing no growth after 24 h). ND: not detectable [47].

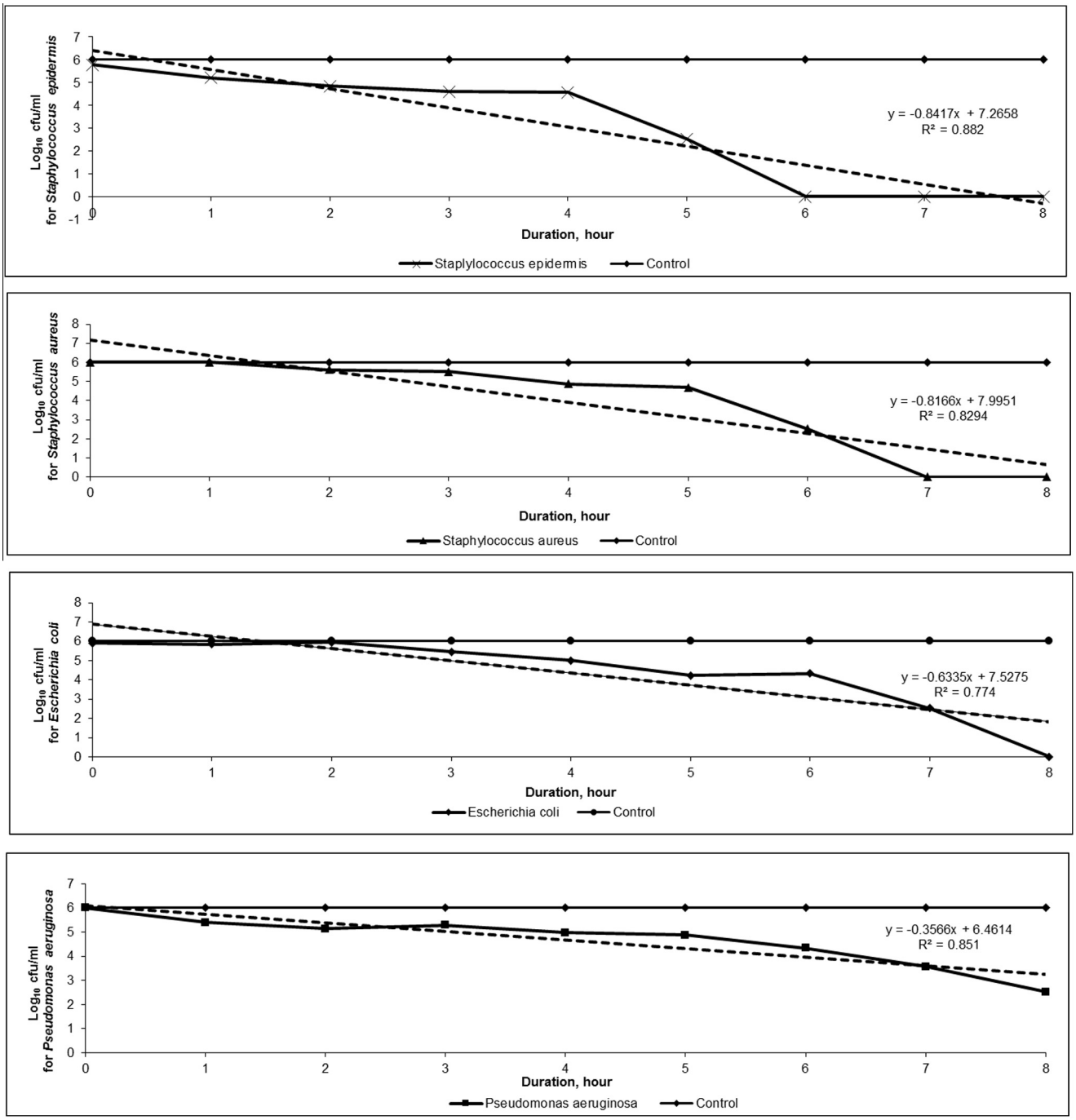


Figure 9 Bactericidal activities of zinc glycerolate against time at 50.0 mg/mL with *S. epidermis*, *S. aureus*, *E. coli* and *P. aeruginosa*. The data represent ±SD of three replicates (error bars not shown as deviations from the mean values are $< 0.1 \times \log_{10}$).

ions, which inhibit several functions in the cell and consequently damage cells. It has also been suggested that zinc binds to the membranes of microorganisms, similar to mammalian cell [35], prolongs the lag phase of the growth cycle and increases the generation time of the organisms so that each organism takes more time to complete cell division [56]. Zinc inhibits multiple activities in the bacterial cell, such as glycolysis, transmembrane proton translocation and acid tolerance [57]. Furthermore, though generally regarded as bacteriostatic, it can have bactericidal effects, particularly when used in combination with other ions, such as fluoride [58], chlorine ions [52] or antibacterial agents like triclosan [59].

In general, Gram-positive aerobic bacteria (*S. aureus* and *S. epidermis*) are more susceptible to zinc ions as compared to Gram-negative aerobic bacteria (*E. coli* and *P. aeruginosa*). Gram-positive bacteria do not have an outer membrane which acts as a barrier whereas Gram-negative bacteria contain an impenetrable outer membrane wall which consists of lipopolysaccharide and protein which envelope the bacteria

Table 4 Time-kill activity of zinc glycerolate at 50.0 mg/mL.

Organism used	D at MIC_a (h)[a]	D^* at MIC_b (h)[b]
Staphylococcus epidermis ATCC 12228	1.19	3.57
Staphylococcus aureus ATCC 6538	1.23	3.68
Escherichia coli ATCC 8739	1.58	4.74
Pseudomonas aeruginosa ATCC 15442	2.81	8.42
Candida albicans ATCC 10231	ND	ND
Aspergillus niger ATCC 9642	ND	ND

ND = not detected [48].
[a] D = time required to achieve 1 $\log_{10}$ (90%) reduction total viable cell count.
[b] D = time required to achieve 3 $\log_{10}$ (99.9%) reduction total viable cell count.

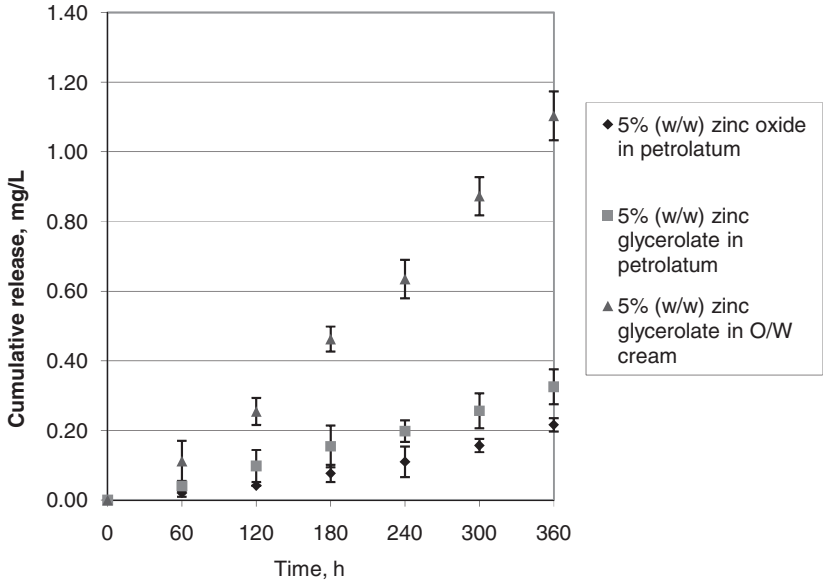


Figure 10 Permeation of zinc ions into PBS in Franz diffusion cell. The data represent ±SD of three replicates.

[60]. On top of that, *P. aeruginosa* is able to produce slime which covers itself, thus exceptionally resistant to chemical agent [61,62]. As noted in the previous section, *P. aeruginosa* has the stronger ability to proliferate and resists the activity of zinc ions better than *E. coli* and this corresponded with the result of previous research done [58]. Zn^{2+} is a also micronutrient for *A. niger* growth [63] and for maintaining the integrity of the cell wall of *P. aeruginosa* [64], hence they were tolerant of high Zn^{2+} concentrations. *P. aeruginosa* can survive longer in damp conditions, and also has a stronger resistance against dryness, ultraviolet and chemical antiseptics such as aldehyde, hydrargyrum and surface-acting agents aside from zinc ions [65]. Heavy metal resistance in microorganisms can occur by a variety of mechanisms, including physical sequestration, exclusion and/or efflux, reduced uptake, detoxification and synthesis of binding proteins. In *Pseudomonas* sp., zinc resistance is inducible. The induction of Zn^{2+} resistance leads to enhanced accumulation of the metal by rapid initial Zn^{2+} uptake followed by gradual stable phase. The uptake of the Zn^{2+} triggered an internal detoxification mechanism that resulted in the extrusion of the excess zinc [51]. In comparison with other divalent heavy metals, such as Cd^{2+}, Pd^{2+}, or Hg^{2+}, a relatively high concentration of Zn^{2+} was required to inhibit growth rates of fungi and to reduce survival of bacteria. This greater tolerance to Zn^{2+} may have reflected the differential sorption of Zn^{2+} to ionogenic sites on the surface of cells [52].

Different interactions between zinc glycerolate/excipient, excipient /membrane and zinc glycerolate/membrane may affect the permeation. In order to assess the mechanism of TRPE permeation through the skin, the influence of K_p, D_m and K_m was investigated particularly between excipient O/W emulsion and petrolatum. As shown in Table 5, D_m and K_m values showed opposite trends. The data obtained pointed out that the D_m alone could not be regarded as a predictive parameter to evaluate zinc ion permeation through the membrane because it takes into account only the lag time but not what happens once the steady state is reached [50]. A more complete frame could be obtained calculating the membrane/excipient partition coefficient, K_m, which was about 11-fold higher for the zinc glycerolate with O/W emulsion than for the zinc glycerolate with petrolatum. Hence, it was noted that

Table 5 Permeation parameters of zinc ion from 5% zinc glycerolate in different excipients namely in O/W emulsion or petrolatum and 5% zinc oxide in petrolatum.

Formulation	J (mg cm^{-2} min^{-1})	t_L (min)	Q_{360} min (mg cm^{-2})	K_p (cm min^{-1})	D_m (cm^2 min^{-1})	K_m
5% (w/w) zinc glycerolate in O/W cream	0.0017	7.29	1.10 ± 0.08	3.47×10^{-8}	2022480.41	2.48×10^{-12}
5% (w/w) zinc glycerolate in petrolatum	0.00051	7.20	0.33 ± 0.02	1.02×10^{-8}	6849747.89	2.17×10^{-13}
5% (w/w) zinc oxide in petrolatum	0.00034	7.62	0.22 ± 0.05	6.80×10^{-9}	10304138.9	9.57×10^{-14}

the inclusion of O/W emulsion as excipient for zinc glycerolate has improved the permeation of zinc ion based on polysulfone membrane model.

Zinc glycerolate has the ability to provide the skin with the mineral zinc and it has potential bioavailability to the epidermis as found in the permeation study using Franz diffusion cell. This was also observed in other study on the skin of rats [17]. Zinc ions from the hydrolysis of zinc glycerolate from moisture in the cream diffused into Franz diffusion cell through polysulfone membrane.

5. Conclusion

Based on these findings, zinc glycerolate has been successfully synthesized. It can be a useful ingredient in the cosmetic and pharmaceutical products for its low irritation to dermal, has sun protective, bactericidal as well as fungistatic properties and able to supplement the skin with zinc nutrient when O/W emulsion was used as the excipient.

Acknowledgements

This research was supported by a Grant from the MPOB. The authors are grateful to Prof. Howard A. Barnes for his assistance in editing this manuscript.

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Please cite this article in press as: M.Y. Cheong et al., Preparation and characterization of zinc glycerolate: UV protection, biological activity and permeation study, Journal of Saudi Chemical Society (2015), http://dx.doi.org/10.1016/j.jscs.2015.09.002